

A Possible Explanation for a Neurotoxic Effect of the Anticancer Agent Oxaliplatin on Neuronal Voltage-Gated Sodium Channels

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Grolleau, Françoise, Laurence Gamelin, Michèle Boisdron-Celle, Bruno Lapiéd, Marcel Pelhate, and Erick Gamelin. A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels. *J Neurophysiol* 85: 2293–2297, 2001. Oxaliplatin, a new widely used anticancer drug, displays frequent, sometimes severe, acute sensory neurotoxicity accompanied by neuromuscular signs that look like the symptoms observed in tetany and myotonia. The whole cell patch-clamp technique was employed to investigate the oxaliplatin effects on the electrophysiological properties of short-term cultured dorsal unpaired median (DUM) neurons isolated from the CNS of the cockroach *Periplaneta americana*. Within the clinical concentration range, oxaliplatin (40–500 μ M), applied intracellularly, decreased the amplitude of the voltage-gated sodium current resulting in a reduction of half the amplitude of the action potential. For comparison, two other platinum derivatives, cisplatin and carboplatin, were found to be ineffective at reducing the sodium current amplitude. In addition, we compared the oxaliplatin action to those of its metabolites dichloro-diaminocyclohexane platinum (dach-Cl₂-platin) and oxalate. Oxalate (500 μ M) was found to be effective, like oxaliplatin, at reducing the inward sodium current amplitude, whereas dach-Cl₂-platin (500 μ M) failed to change the current amplitude. Interestingly, the effect of oxalate or oxaliplatin could be mimicked by using intracellularly applied 10 mM bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA), known as chelator of calcium ions. We concluded that oxaliplatin was capable of altering the voltage-gated sodium channels through a pathway involving calcium ions probably immobilized by its metabolite oxalate. The medical interest of preventing acute neurotoxic side effects of oxaliplatin by infusing Ca²⁺ and Mg²⁺ is discussed.

INTRODUCTION

Oxaliplatin is a new diammine cyclohexane platinum derivative that is active in several solid tumor types, especially in some cisplatin/carboplatin refractory diseases such as colorectal cancer (Louvét et al. 1996; Machover et al. 1996). Cisplatin was the first platinum derivative used in cancer treatment, but its use was limited due to severe neurotoxic side effects that emerged as dose-limiting (Gregg 1992; Mollman 1990; Moll-

man et al. 1988). The initial symptoms of cisplatin neurotoxicity were paresthesias, numbness, and tinglings that appeared at cumulative dose of 300–400 mg/m² (Mollman et al. 1988; van der Hoop et al. 1990). At 400–600 mg/m², cisplatin induced a peripheral sensory neuropathy including loss of vibratory sensation, loss of deep tendon reflexes and proprioceptive sensory ataxia. Sensory nerve conduction was altered, whereas motor conduction velocity remained normal (Hamers et al. 1991; Mollman 1990).

Substantial progress has been made in cancer therapy with the introduction of a new generation of platinum derivatives including oxaliplatin and carboplatin. Oxaliplatin is active in several solid tumor types, especially in some cisplatin-resistant cancers (Machover et al. 1996) and is better tolerated than cisplatin, particularly in terms of renal toxicity (Cvitkovic and Bekradda 1999; Extra et al. 1998). In return, a peripheral sensory neuropathy is related to the cumulative dose of oxaliplatin, producing symptoms that resembled many of those observed during cisplatin treatment. Unlike cisplatin, this neurotoxicity is moderate and generally reversible. However, in almost 90% of the patients, oxaliplatin induces also a unique acute peripheral sensory and motor toxicity that occurs often during or within hours after oxaliplatin infusion (Raymond et al. 1998a,b). This toxicity shows a rapid onset and was characterized by acral paresthesia or cold-related dysesthesia affecting the perioral and laryngo-pharyngeal areas and the upper and lower limbs. Motor component is characterized by tetanic spasms, myotonia, cramps, prolonged muscular tense, muscular fasciculations, affecting legs, thighs, hands, and jaws, hampering movements.

Because oxaliplatin-induced neurotoxicity causes significant discomfort, alters patient quality of life, and may be accompanied by significant disability, effort could be made to optimize treatment of colorectal cancers. Based on different symptoms observed during oxaliplatin infusion, we speculated that this compound or one of its metabolites, dichloro-diaminocyclohexane platinum (i.e., dach-Cl₂-platin) or oxalate, may alter the properties of the voltage-gated sodium channels known to be involved in the action potential generation. In addition, many

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of the neurological effects induced by oxaliplatin could be strongly attenuated by pre- and posttreatment with Ca^{2+} and Mg^{2+} infusion (Lainé-Cessac et al. 1998), suggesting a mode of action involving a Ca^{2+} -dependent mechanism by oxaliplatin itself, or its metabolite oxalate, which is well known to be a chelator of calcium ions in biochemistry and toxicology (Jacobsen and McMartin 1986). To verify our hypothesis, electrophysiological studies have been carried out on cockroach dorsal unpaired median (DUM) neurons. Cockroach neuronal preparations are commonly used as biomedical models for vertebrates (Pelhate et al. 1990), and DUM neurons are, furthermore, electrophysiologically well characterized since most of the biophysical and pharmacological properties of ionic currents underlying their spontaneous action potentials have been established by using the patch-clamp technique (Grolleau and Lapiéd 2000).

In this study, we have tested, on DUM neurons, oxaliplatin, two of its metabolites, dach-platin and oxalate, and, for comparative purposes, other platinum derivatives such as cisplatin and carboplatin. We conclude that the inhibitory effect of oxaliplatin on the voltage-gated sodium current was mainly mediated through a calcium ion immobilization by oxalate residue.

METHODS

Adult male cockroaches, *Periplaneta americana*, were taken from our laboratory colonies, which were maintained under standard conditions (28°C, photoperiod of 12 h light/12 h dark). The ganglionic ventral nerve cord and its terminal abdominal ganglion (TAG) were carefully dissected under a binocular microscope and placed in normal cockroach saline containing (in mM) 200 NaCl, 3.1 KCl, 5 CaCl_2 , 4 MgCl_2 , 50 sucrose, and 10 *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES); pH was adjusted to 7.4 with NaOH. Isolation of adult DUM neuron cell bodies were performed under sterile conditions using enzymatic digestion and mechanical dissociation of the median parts of the TAG as previously described (Grolleau and Lapiéd 1996; Lapiéd et al. 1990). The isolated neuron cell bodies were used for recordings 24 h after dissociation.

We used the patch-clamp technique in the whole cell recording configuration (Hamill et al. 1981) to record voltage-gated sodium currents (voltage-clamp mode) and action potentials (current-clamp mode). Signals were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from borosilicate glass capillary tubes (Clark Electromedical Instruments, Reading, UK) with a PP-83 electrode puller (Narishige, Japan) and had resistances of 0.9–1.2 M Ω when filled with the pipette solution (see composition below). The liquid junction potential between bath and internal solutions was always corrected before the formation of a gigaohm seal (>2 G Ω). For voltage-clamp experiments, step voltage pulses were generated by a programmable stimulator (SMP 310, Biologic, Claix, France) or by an IBM Pentium 100 computer with software control pClamp (version 6.0.3, Axon Instruments) connected to a 125-kHz labmaster DMA acquisition system (TL-1-125 interface, Axon Instruments). Cells were clamped at a holding potential of -90 mV, and 30-ms test pulses were applied from the holding potential at a frequency of 0.3 Hz. Cells that exhibited an “all-or-none” current-voltage relationship or a stepwise activation of currents were presumed to be inadequately clamped and were discarded. It was usually possible to compensate for up to 70% of the series resistance without introducing oscillations into the recorded current. Although leak and capacitive currents were compensated electronically at the beginning of each experiment, subtraction of residual capacitive and leakage currents was performed with an on-line P/6 protocol provided by

pClamp. Data were displayed on digital oscilloscope (310 Nicolet Instrument, Madison, WI) and stored on the hard disk of the computer (sampling frequency 30.3 kHz) for subsequent off-line analysis. The extracellular solution superfusing the cell used to record inward sodium currents contained (in mM) 100 NaCl, 100 tetraethylammonium chloride (TEA-Cl), 3.1 KCl, 2 CaCl_2 , 7 MgCl_2 , 1 CdCl_2 , 5 4-aminopyridine, and 10 HEPES; pH was adjusted to 7.4 with TEA-OH. Patch electrodes were filled with an internal solution containing (in mM) 90 CsCl, 80 CsF, 15 NaCl, 1 MgCl_2 , 2 ATP-Mg, 5 ethyleneglycol-bis-(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10 HEPES; pH was adjusted to 7.4 with CsOH. The bathing solution used to record inward calcium currents contained (in mM) 100 Choline chloride, 3.1 KCl, 4 MgCl_2 , 5 CaCl_2 , 100 TEA-Cl, 5 4-aminopyridine, and 10 mM HEPES; pH was adjusted to 7.4 with TEA-OH. The internal pipette solution contained (in mM) 155 CsCl, 10 CsF, 10 NaCl, 0.5 CaCl_2 , 10 EGTA, 3 ATP-Mg, 0.2 GTP- Na_2 , and 20 HEPES; pH value was adjusted to 7.4 with CsOH.

For current-clamp recordings, action potentials were evoked by applying a 50-ms depolarizing current pulse of 0.6–0.8 nA at 0.5 Hz with a programmable stimulator (SMP 310, Biologic). Signals were displayed on a digital oscilloscope (Nicolet) and stored on a DTR 1202 (Biologic). The bathing solution contained (in mM) 200 NaCl, 3.1 KCl, 5 CaCl_2 , 4 MgCl_2 , and 10 HEPES; pH was adjusted to 7.4 with NaOH. The recording electrode was filled with (in mM) 160 potassium aspartate, 10 KF, 10 NaCl, 0.5 CaCl_2 , 10 EGTA, 1 MgCl_2 , 1 ATP-Mg, and 10 HEPES; pH was adjusted to 7.4 with KOH. All compounds were purchased from Sigma Chemicals (L'isle d'Abeau Chesnes, France) except oxaliplatin, which was obtained from Sanofi. Experiments were carried out at room temperature (20°C). Data, when quantified, were expressed as means \pm SE.

RESULTS

Effect of oxaliplatin on DUM neuron voltage-dependent sodium current

The blocking effect of oxaliplatin on voltage-dependent sodium current was investigated on DUM neurons in voltage- and current-clamp modes. The main advantage of the whole cell recording of the patch-clamp technique is the possibility to apply oxaliplatin intracellularly (i.e., through the intrapipette solution) or extracellularly (i.e., through the bathing solution superfusing the cell body). Figure 1A shows typical examples of inward sodium currents from isolated DUM neuron cell body in response to a 30-ms depolarizing step to -10 mV applied from a holding potential of -90 mV. After 20 min of exposure, 100 μM oxaliplatin added in the extracellular solution superfusing the cell produced a slight reduction of the maximum peak sodium current by $16.7 \pm 5.8\%$ (mean \pm SE, $n = 6$). By contrast, intracellular application of oxaliplatin at the same concentration during 20 min caused a marked inhibition of the current amplitude ($52.8 \pm 3.3\%$, $n = 5$, Fig. 1, A and B), indicating that oxaliplatin is much more active when applied on the intracellular face of the membrane. It should be noted that no significant change of the leakage current, measured when hyperpolarizing voltage steps (130 ms in duration) in 10-mV increments were applied from a holding potential of -90 mV, was observed during oxaliplatin application (Fig. 1C). Interestingly, such effect of oxaliplatin on sodium inward current was reached only on 7 of 11 neurons tested since oxaliplatin was found active particularly on the DUM neuron cell bodies exhibiting inward sodium current with a sustained component (Fig. 2, Aa and Ab). By contrast, oxaliplatin induced lower effect on fully inactivated inward sodium current

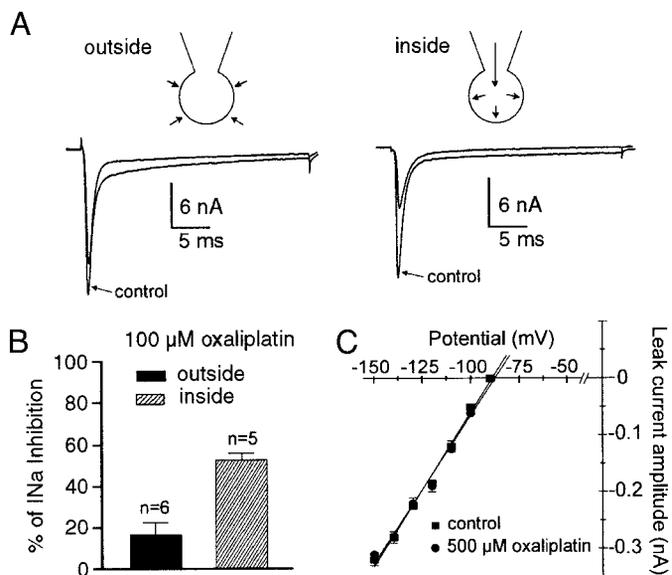


FIG. 1. Effect of oxaliplatin (100 μ M) on voltage-gated Na⁺ current in isolated dorsal unpaired median (DUM) neuron cell bodies. **A**: inward Na⁺ currents were elicited with a 30-ms depolarizing pulse to -10 mV from a holding potential of -90 mV. Currents were capacity and leak corrected. *Left trace* shows that oxaliplatin had a slight effect on the peak current after 20 min of bath application. By contrast, when oxaliplatin was applied intracellularly through the pipette solution, the current was markedly suppressed (*right trace*). **B**: comparative histogram of the percentage of oxaliplatin inhibition measured after 20 min in both conditions. Note that oxaliplatin was at least 4 times more potent at reducing the peak Na⁺ current amplitude when applied intracellularly. **C**: current-voltage relationship of inward leakage current amplitudes obtained in control (■) and with 500 μ M oxaliplatin added in the pipette solution (●). The 130-ms duration hyperpolarizing potentials were applied in 10-mV increments. Note that no significant change of the leakage current ($n = 4$) was observed after 25 min of intracellular application of oxaliplatin.

(Fig. 2*Ab*). When oxaliplatin was active, the time course of its effect (Fig. 2*B*) showed that the current amplitude inhibition was progressive during the first 10 min following the whole cell establishment and then stabilized after about 12–15 min. The intracellular oxaliplatin-induced inhibition of the maximum peak sodium current amplitude was dose dependent.

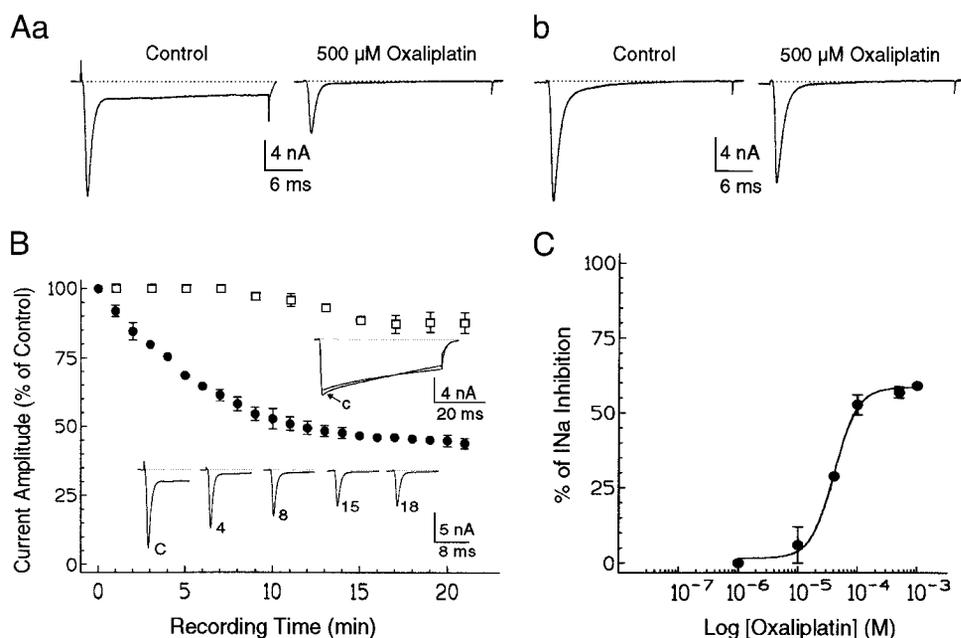
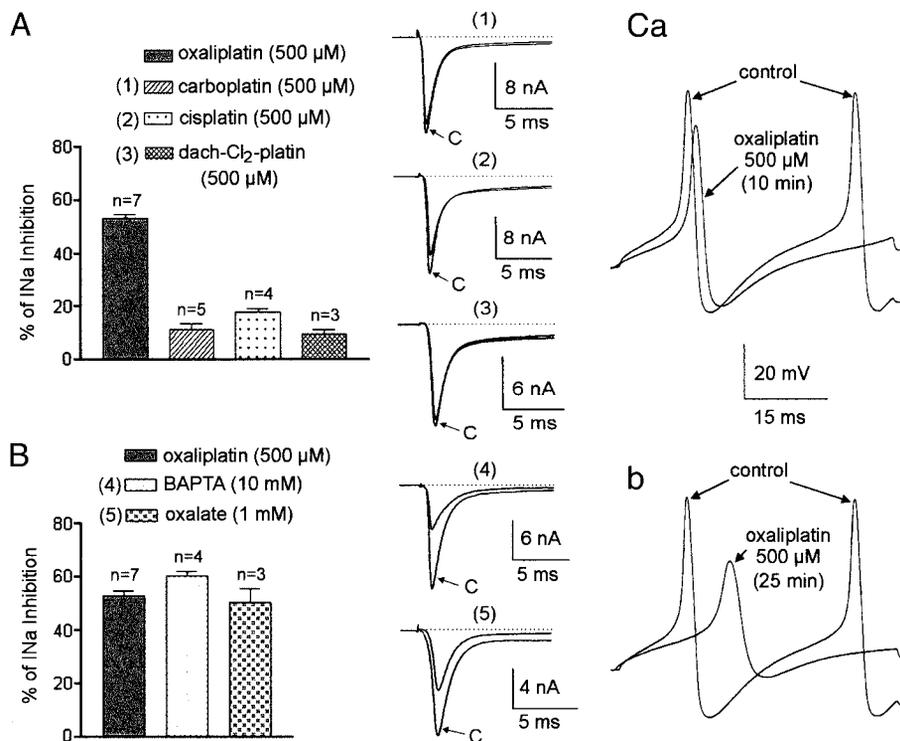


FIG. 2. **A**: current traces illustrating different effects induced by oxaliplatin on inward Na⁺ current that exhibited a sustained component (*a*) and on completely inactivated inward Na⁺ current (*b*). Note that oxaliplatin reduced preferentially the inward Na⁺ current amplitude that did not fully inactivate. **B**: superimposed time courses of the effect of oxaliplatin (500 μ M in the pipette) on voltage-dependent peak Na⁺ (●) and Ca²⁺ (□) currents. *Inset*: top traces illustrate typical examples of Ca²⁺ currents recorded in response to a 90-mV depolarizing pulse from a HP -100 mV, immediately (*C*) and 20 min after establishment of the whole cell patch-clamp configuration. *Bottom traces* show the time-dependent reduction of a control (*C*) Na⁺ current evoked by a 30-ms voltage step from -90 to -10 mV with 500 μ M in the pipette. **C**: dose-dependent inhibition plot for oxaliplatin on peak Na⁺ current. Data points indicate mean \pm SE %reduction of the current after 20 min of oxaliplatin application at various concentrations.

When mean values for percentage of inhibition were plotted against the logarithm of oxaliplatin concentrations (Fig. 2*C*), a sigmoid curve was obtained. The solid line corresponds to the best fit (correlation coefficient $r = 0.998$) through the mean data points ($n = 3-6$) according to a four-parameter logistic equation: $Y = Y_{\max}/[1 + (IC_{50}/\text{Conc.})^n]$, where Y_{\max} is the maximum value of percentage of inhibition, IC_{50} is the concentration that produced 50% inhibition of the peak inward sodium current, and n is the Hill coefficient (or slope factor). The IC_{50} value and slope factor estimated for oxaliplatin was 42 μ M and 2.25, respectively. It is important to note that the sodium current block was not complete and the maximum blocking effect was only $59.2 \pm 1.6\%$ ($n = 3$) with 1 mM oxaliplatin. For comparison, the well-known selective sodium channel blockers, tetrodotoxin and saxitoxin completely block the DUM neuron inward sodium current at lower concentration (Lapied et al. 1990). To ensure whether oxaliplatin acted selectively or not on voltage-dependent sodium channels, voltage-clamp experiments were also performed on the high-voltage-activated (HVA) inward calcium current previously characterized in the same preparation (Grolleau and Lapied 1996). Oxaliplatin applied at 500 μ M, which gave maximum effect on the inward sodium current, failed to block DUM neuron HVA calcium current elicited by a 100-ms depolarizing pulse from a holding potential of -100 mV (Fig. 2*B*, *inset*).

Effects of other platinum derivatives

We compared oxaliplatin action to those of different platinum derivatives such as cisplatin, carboplatin, and dach-Cl₂-platin. Dach-Cl₂-platin is one of the metabolites of oxaliplatin. It is considered as the toxic and active metabolite that ultimately reacts with DNA, and it is released when oxalate moiety is displaced intracellularly by bicarbonate ions (Cvitkovic and Bekradda 1999; Mauldin et al. 1988; Screnci et al. 1997). Dach-Cl₂-platin as well as cisplatin and carboplatin was found much less potent than oxaliplatin at reducing the voltage-dependent inward sodium current (Fig. 3*A*) when applied intracellularly at 500 μ M. By contrast, when the second me-



tabolite of oxaliplatin, oxalate, was added in the pipette solution at a concentration of 500 μM , the inward sodium current amplitude was reduced by $36 \pm 2\%$ ($n = 3$). Like oxaliplatin, the effect of oxalate was dose dependent. When tested at 1 mM, oxalate reduced the current by $50.5 \pm 7.5\%$ ($n = 3$, Fig. 3B). Since oxalate is known to be capable of immobilizing calcium ions, we performed an additional set of experiments to check whether or not its effect could be mimicked by the use of bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) known to be a strong calcium ion chelator. As illustrated in Fig. 3B, the addition of high concentration of BAPTA (10 mM) in the pipette solution decreased by $60.3 \pm 3.3\%$ ($n = 4$) the amplitude of the inward sodium current. The results, summarized on the histogram in Fig. 3B, indicate that calcium buffering by either oxalate or BAPTA reduced the sodium current amplitude in the same order of magnitude as oxaliplatin. This suggests that oxaliplatin effect may be mediated by a decrease in the intracellular calcium concentration.

Functional significance

The somata of DUM neurons maintained in short-term culture were capable of generating spontaneous or triggered overshooting sodium-dependent action potentials (Grolleau and Lapied 2000). Action potentials could be elicited by injecting a depolarizing current pulse (0.8 nA for 50 ms). Superimposed evoked action potentials recorded in control condition or with intracellularly applied 500 μM oxaliplatin are illustrated in Fig. 3C. After 10 min (Fig. 3Ca), oxaliplatin reduced the spike amplitude by 19.4%. This blocking effect appeared progressively without significant change in the posthyperpolarization amplitude. By contrast, oxaliplatin prolonged spike interval and decreased the slope of prepolarization. For longer oxaliplatin application (i.e., 25 min), we observed an important reduction of both depolarizing phase and posthyperpolarization

associated with an increase in action potential duration (Fig. 3Cb). These two last effects reflected the oxalate-induced chelation of intracellular calcium, which thereby inhibited calcium-activated potassium channels previously characterized in DUM neurons (Grolleau and Lapied 2000).

DISCUSSION

This is the first published study reporting an inhibitory effect of oxaliplatin on voltage-gated sodium current. As in many excitable cells, the activation of the voltage-dependent sodium current controls the rising phase of the action potential in DUM neurons (Grolleau and Lapied 2000). Oxaliplatin reduced the spike amplitude by altering voltage-dependent sodium channels. In these regards, acute oxaliplatin toxicity is thought to have neurological origin like those observed during tetrodotoxin, which was mainly based on blocking effect of TTX on the voltage-gated sodium channels (Hille 1992; Yang et al. 1996).

We have compared oxaliplatin action to those of different platinum derivatives used as cytotoxic drugs, such as cisplatin, the first available platinum derivative, generating chronic and cumulative irreversible peripheral neuropathy, and carboplatin, which is nonneurotoxic. None of them cause acute peripheral neuropathy, and none of them has been found to alter the sodium current amplitude on DUM neurons. However, the knowledge of the oxaliplatin metabolism helped us to better understand the oxaliplatin-induced neurotoxicity. Biotransformation of oxaliplatin gives two major metabolites, dach-Cl₂-platin and oxalate ions (Mauldin et al. 1988; Scenci et al. 1997). Oxalate is well known, in biochemistry and toxicology, to be a strong calcium chelator and to be responsible, for instance in ethylene glycol poisonings, of tetanic spasms, and muscular hyper-excitability because it rapidly precipitates with Ca^{2+} ions in various tissues (Jacobsen and McMartin 1986).

FIG. 3. *A*: effect of oxaliplatin on DUM neuron inward sodium current compared with other platinum derivatives carboplatin, cisplatin and dach-Cl₂-platin. The histogram shows the mean effects \pm SE ($n = 3-7$) measured on the peak Na^+ current for each compound after 20 min of intracellular application. *B*: comparative histogram of the percentage of the peak Na^+ current inhibition produced by intracellularly applied bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) and oxalate. The current traces shown in *A* and *B* were the corresponding capacity and leak-corrected inward Na^+ currents elicited with a 30-ms depolarizing pulse to -10 mV from a holding potential of -90 mV and recorded immediately (*C*) and 20 min after establishment of the whole cell configuration with different drugs (1, 2, 3, 4, 5) in the pipette. *C*: effect of oxaliplatin on triggered action potentials evoked by a depolarizing constant 50-ms pulse of 0.8 nA. Control action potentials (control) were recorded within the 1st minute after establishment of the whole cell configuration. The superimposed trace corresponds to the action potential recorded after 10 min (*a*) and 25 min (*b*) with 500 μM oxaliplatin in the pipette solution.

Our results show that, unlike dach-Cl₂-platin, oxalate is capable of producing the same inhibitory effects than those obtained with oxaliplatin or BAPTA used as pharmacological tools to immobilize Ca²⁺ ions. This suggests that oxaliplatin blocks DUM neuron voltage-gated sodium channel via a chelation of calcium ions through the action of its metabolite, oxalate. In this condition, at least two mechanisms accounting for oxaliplatin neurotoxicity should be proposed: 1) calcium-sensitive voltage-gated sodium channels may exist and could be directly affected following calcium chelation by oxalate or 2) oxalate may affect indirectly the voltage-gated sodium channels through an intracellular Ca²⁺-dependent regulatory mechanism.

In conclusion, this study indicates that neuronal damage produced by oxaliplatin may result in part from the effects of this drug on voltage-gated sodium channels and chronic oxaliplatin-induced neuropathy could be the long-term consequence of its acute toxicity. On the other hand, cisplatin-induced neuropathy is classically reported to be due to a very long-term platinum retention in deep compartments, especially in neuronal tissue together with a progressive accumulation (Gamelin et al. 1995). By contrast, oxaliplatin has a completely different pharmacokinetic profile and does not accumulate in plasma with repeated chemotherapy cycles (Gamelin et al. 1997). In fact, our electrophysiological results are consistent with previous clinical observations (Lainé-Cessac et al. 1998), and immediate oxalate control could be expected to prevent some of neurological effects observed during and after oxaliplatin treatment. When Ca²⁺ and Mg²⁺ were infused to patients before and after oxaliplatin administration, oxaliplatin-induced acute neurotoxicity was highly reduced, becoming lower than 10% of grade 2 and 3 in 40 patients (data not shown). A national multicentric double blind trial, from the "Fondation Française de Cancérologie Digestive" is carried out, with the purpose to confirm the effect of Ca²⁺ and Mg²⁺ infusion for preventing acute neurotoxicity in patients treated with oxaliplatin.

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